A Novel Method for the Visualization of Microtubules in Plant Tissues

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This paper describes new improved techniques to visualize microtubules *in situ* by immunofluorescence microscopy of thick plant tissue sections. The essential components of the technique include slow infiltration of the fixed tissue in 1.5-2 M sucrose (which acts as a cryoprotectant and gives physical support to the tissue during frozen sectioning) and post-infiltration embedding in 10% gelatine in 1.5 M sucrose which provides further support to the tissue and prevents fragmentation during sectioning. A new method for coating glass slides with poly-L-lysine is also described, which dramatically improves the success of section-adherence to the slides during immunostaining. Examples are given of successful microtubule localization in root tissue of *Pisum sativum* using these methods. This improved technique provides a reliable investigative tool with which to elucidate the mechanisms underlying the cytoskeletal control of plant cell growth and morphogenesis.

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INTRODUCTION

Microtubules are filamentous assemblages of tubulin proteins which form a major component of the complex cytoskeletal network that extends throughout the cytoplasm of plant and animal cells. The cytoskeleton as a whole, and microtubules in particular, play a myriad of roles in the regulation of cell and organismal development. For instance, the cytoskeleton is directly responsible for motility and muscle contraction in animal cells and it provides the machinery for intracellular organelle traffic in both plant and animal cells (Alberts et al., 1989). In plant tissues, microtubules are of particular importance since they are known to play a specific role in orchestrating axes of cell expansion and cell division and can thus directly influence spatial development and morphogenesis (e.g. see reviews by Hardham, 1982; Seagull, 1989).

To understand exactly how microtubules can orchestrate such precise spatial and developmental control in cells of whole plant tissues it is essential to be able to examine microtubule arrays of many cells *in situ*. It first became possible to observe microtubules in plant cells in 1963, due to improved cytochemical fixation techniques (Ledbetter and Porter, 1963) and these dynamic tubular structures have since been identified as a major element of the cortical cytoplasm of most higher plant cells (Hepler and Palevitz, 1974; Hepler, 1976; Filner and Yadav, 1979; Gunning and Hardham, 1982; Lloyd, 1987; Seagull, 1989). Although electron microscopy has yielded much ultrastructural information on plant cytoskeletal organization (e.g. Ledbetter and Porter, 1970; Hardham and Gunning, 1978), it allows examination of relatively few cells at a time, and usually produces two-dimensional information on only a portion of an entire cell. In contrast to this, the technique of immunofluorescence microscopy (using anti-tubulin to visualize microtubules) allows the examination of three-dimensional cytoskeletal arrays in cells across large areas of tissue (e.g. Wick et al., 1989).

Various immunofluorescence methods are currently in use for the visualization of microtubules in plant tissues, but there are substantial problems associated with each

of these methods for immunolabelling in sections of large areas of tissue (discussed below). This paper describes the development of a successful new protocol for immunofluorescence localization of microtubules in plant tissues.

Materials and Methods

Plant Material

The root tips of *Pisum sativum* L. var. Greenfeast were used as a representative higher plant tissue for microtubule localization. Seeds of *P. sativum* (gift from Yates and Co. Milperra, N.S.W., Australia) were surface sterilized in 6% (W/V) NaClO for 5 minutes, rinsed four times in sterile distilled water (dH_2O), and aseptically germinated in petri dishes containing 1% agar in ¼ strength modified Hoagland's solution (0.38 mM $MgSO_4.7H_2O$; 0.34 mM $NaH_2PO_4.2H_2O$; 0.07 mM $Ca(NO_3)_2.4H_2O$; 1.00 mM (KNO_3). 4 day old seedlings grown at $20 \pm 2°C$ in the dark were used in all experiments.

Tissue Fixation

Satisfactory tissue fixation was achieved with a standard fixation schedule of 1 hour's fixation in 3% paraformaldehyde (Sigma) in microtubule stabilizing buffer (MSB): 0.1 M 1,4-piperazine-diethanesulphonic acid (PIPES) buffer; 1 mM ($MgSO_4$; 2mM ethyleneglycol-bis-(B-aminoethyllether)-N,N,N,N^1-tetraacetic acid (EGTA); adjusted to pH 6.9 with 1 M NaOH). Various additions and modifications were attempted, such as 0.05% or 0.1% Triton-X 100 (to enhance antibody penetration through the tissue section) and 1%, 5%, or 10% dimethylsulphoxide (DMSO) to improve microtubule preservation (Schroeder et al., 1985). The best tissue and microtubule preservation was achieved with the addition of 0.05% Triton-X and 10% DMSO to the fixtive.

Tissue Infiltration

Previously, immunofluorescence techniques have been used to visualize microtubules in cells within tissue that has been embedded and sectioned in polyethylene glycol (PEG) (Tiwari et al., 1984; Derksen et al., 1986) or butyl-methyl methacrylate (BMM) (Gubler, 1989), or cryosectioned in frozen drops of dH_2O (Hogetsu and Oshima, 1986; Sakaguchi et al., 1988) or Tissue-Tek (Cleary and Hardham, 1989). Each of these methods was attempted with pea root tissue, and none was satisfactory: obtaining reasonable sections was difficult, particularly with large areas of more mature root tissue, for roots embedded in PEG (microtome sections), or frozen drops of dH_2O or Tissue-Tek (cryosections), in which tissue tended to fragment during sectioning. An additional difficulty with the PEG embedding was that it was very difficult to assess the orientation of the tissue in the opaque PEG block. This is important because it is essential to know exactly which plane the tissue is sectioned in, so as to avoid possible mis-interpretation of microtubule orientation. BMM-embedded roots sectioned well, but immunofluorescence staining was unsuccessful either directly following the Gubler (1989) method, or trying modifications of it (e.g. different fixation solutions, tubulin antibodies or staining times), and so this approach was eventually abandoned. More recent modifications of this technique (Baskin, et al. (in press)) have improved immunostaining of tissue embedded in BMM, although the limitation of thin sectioning with this technique, remains.

It was necessary, therefore, to develop a different embedding protocol which could yield satisfactory sections of tissue for immunofluorescence tubulin staining. The problems of tissue collapse experienced with cryosections of root tissue embedded in frozen drops of dH_2O or Tissue-Tek were reduced greatly, but not completely, by

embedding and cryosectioning in 1.5-2.0 M sucrose, which acts as a cryoprotectant and a support for the tissue during sectioning (Hawes, 1988). However, a critical step which virtually eliminated this problem is post-infiltration embedding with 10% gelatine in 1.5 M sucrose at 37°C (after Hawes, 1988). The effects of various infiltration times were assessed and ease of sectioning was maximized after 2-3 hours gelatine infiltration.

Final Fixation and Infiltration Schedule

Root tips (about 3-5 mm in length) were cut in a drop of fresh fixative (3% formaldehyde, 0.05% Triton-X and 10% DMSO in MSB). After fixation for 1 hour at RT, the tissue was rinsed twice with MSB and perfused with sucrose, increasing the concentration slowly (over a day) until it reached 2 M. Infiltration was completed overnight while roots were left in 2 M sucrose at 4°C. Root segments were then incubated in 10% (w/v) gelatine (BDH Poole, England) in 1.5 M sucrose at 37°C for 2 hours, and frozen onto cryo-stubs in a drop of 1.5 M sucrose. These stubs could be stored in liquid nitrogen before sectioning.

Sectioning: Preliminary Studies

Using a Bright Starlet 2212 Cryostat, cryosections were taken at thicknesses of 2-12 μm and best results were obtained at 8-10 μm. The temperature of the whole cryo-chamber was maintained at -37°C. Additionally, it was useful to control the local temperature of the sucrose block and the knife blade, which could be cooled using a freezing spray (Histospray) or warmed by gentle breathing. The ideal combination was to have the knife blade colder than the face of the sucrose block.

One of the most persistent problems encountered in developing the techniques for microtubule visualization was keeping cryosections well adhered to the surface of the slides, since these sizeable sections (approximately 3 mm x 2 mm x 10 μm) could easily be washed away during the immunofluorescence staining schedule. The commonly used methods, namely: (i) applying a small amount of poly-L-lysine (1 mg ml^{-1}) to each slide well and heating the slide briefly through a flame during application (Adrienne Hardham, pers. comm.); (ii) pipetting 10 μL of poly-L-lysine (1 mg ml^{-1}) to each slide well, leaving covered for 1 hour in a petri dish and then rinsing in dH_2O, and (iii) coating slides with a subbing solution containing gelatin, chrome alum and Teepol (Hawes, 1988), were all found to be unsatisfactory. A final technique which was more successful in keeping the large cryosections secure on the glass slides, was to place slides face-down on a piece of parafilm (hydrophobic laboratory plastic) containing drops of a high molecular weight (390,000 g mol^{-1}) poly-L-lysine (Sigma; 5 mg ml^{-1}) for 2-3 hours and then wash in dH_2O (Beatrice Satiat-Jeunemaitre, pers. comm.). Finally, slides are heated (in a glass slide rack covered with foil) in an oven at 70°C for at least 2 hours. Possibly this heat step is effective due to some form of heat activation of the poly-L-lysine cation.

Sectioning: Final Schedule

Root segments were frozen in a drop of 1.5 M sucrose, and longitudinal sections (8-10 μm) were cut at approximately -37°C. All cryosections were made in the longitudinal plane of the root. Sections were picked up in a drop of 1.5 M sucrose in a wire loop and touched onto the surface of a poly-L-lysine coated multiwell slide. Alcohol cleaned multiwell slides were prepared by placing face down onto a piece of parafilm containing 4 x 15 μL drops (per slide) of high molecular weight poly-l-lysine (390,000 g mol^{-1}) at a concentration of 5 mg ml^{-1}, for at least 2 hours. It was essential to use freshly prepared poly-L-lysine solutions. Slides were rinsed thoroughly in double-distilled H_2O

and heated at approximately 70°C for at least 2 hours. Tissue sections on slides stored in the dark at 4°C retained immunoreactivity for many weeks.

Tubulin Immunolabelling

Sucrose was washed out of the root cryosections by rinsing (2 x 10 min) in MSB. The primary antibody used was YOL 1/34, a monoclonal rat IgG raised against yeast tubulin (Serotec Ltd., Bicester, England) at a dilution of 1:200 (in MSB) applied to each section for 1.5 hours. A rinse in MSB (2 x 10 min) preceded application of the secondary antibody, fluorescein isothiocyanate (FITC)-labelled sheep anti-rat IgG (Serotec) diluted 1:40 (in MSB), for 1-2 hours. Recently, we have found that application of the secondary antibody overnight in a moist chamber at 35°C, further improves tubulin staining. Cryosections were given final rinses (3 x 10 min) in MSB and double-distilled H_2O (1 x 10 min) before mounting in Citifluor (Citifluor Ltd., City University, London, England), a glycerol-PBS solution containing *para*-phenylene-diamine which retards quenching of the fluorescence. All staining procedures were carried out at room temperature. In later experiments, fish emulsion (gelatin from cold water fish skin; Sigma) was added (1% in MSB) to all solutions after the primary antibody, which reduced non-specific background staining (Satiat-Jeunemaitre, pers. comm.).

Microscopy

Sections were examined with a Zeiss Axiophot epifluorescence photomicroscope. FITC-labelled microtubules were observed using the Zeiss filter set #487917 (excitation blue H485 SB). Plan Neofluar objectives 2.5/0.75, 10/0.30, 40/0.75 and 100/1.30 (oil) and Plan Apochromat 63/1.40 (oil) objectives were used. Black and white photographs were taken on either Ilford HP5 400 ASA (developed in Ilford Microphen developer) or Ilford Tech-pan 100 ASA (developed in Ilfotech film developer).

Results

This new protocol of sucrose-embedding, gelatine-infiltration and poly-L-lysine slide preparation for cryosectioning and immunostaining microtubules in plant tissues was particularly successful in enabling a clear examination of microtubule alignment in cells *in situ*. This is illustrated in the immunofluorescence micrographs (Figs. 1 and 2) of cells in pea root tissue. Note that all micrographs are oriented with the vertical axis parallel to the longitudinal axis of the root, which is also the axis of reference for microtubule orientations (for example, 'transverse' microtubules are aligned perpendicular to the longitudinal axis of the root).

Figure 1 shows microtubules in cells from the zones of maturation (A, B), elongation (C) and division (D) and these micrographs indicate how this technique allows the various arrays and alignments of microtubules in representative cells from these different morphological zones to be distinguished. In all non-dividing cells, it is apparent that cortical microtubules are aligned in parallel arrays. In very mature cells which have ceased elongating, microtubules can be seen in an oblique orientation (Figs 1A, B). However, in cells of the elongating zone and meristematic zone, a distinctly transverse alignment of cortical microtubules can be seen (Figs. 1C and 1D, arrow). Microtubules constitute the spindle, phragmoplast and the pre-prophase band in mitotic cells and these arrays were also clearly visualized using this technique. Figure 1D illustrates the appearance of cells with a pre-prophase band in section (e.g. single arrowheads). This specialized array of microtubules is found only in plant cells, exists transiently before and sometimes into early prophase as a tight girdle around the cell,

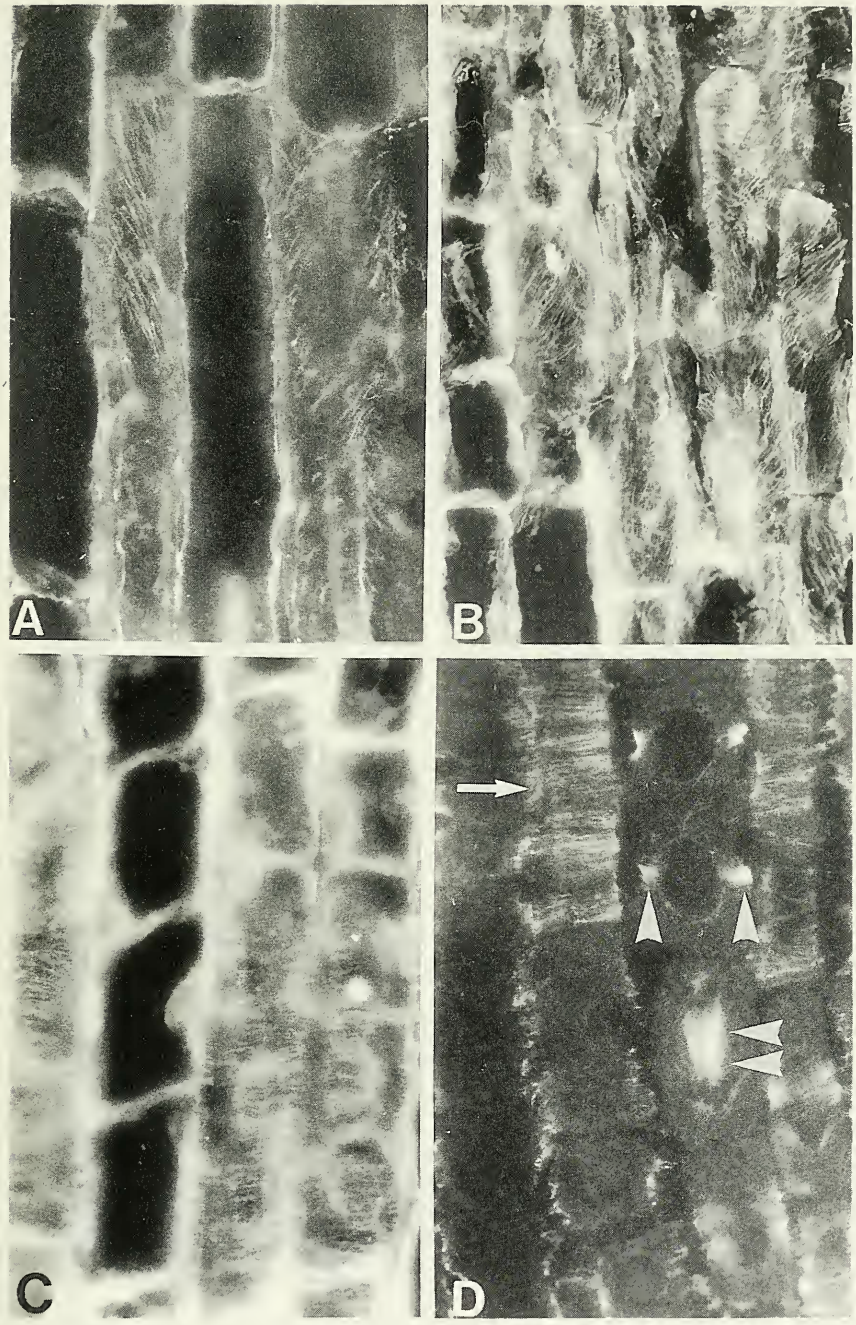

Fig. 1. Immunofluorescence micrographs of immunolabelled microtubules in *P. sativum* root cells. Microtubules are shown in cells at approximately 4.5 cm (**A**), 3.5 cm (**B**), 2.5 cm (**C**) and 1.5 cm (**D**) from the root tip. Cells in the zone of differentiation (**A-B**) exhibit obliquely oriented microtubules while microtubules are transversely aligned in cells in the zone of elongation (**C**). In the meristematic zone (**D**), transverse microtubule arrays are visible in interphase cells (e.g. arrow) and in meristematic cells, pre-prophase bands of microtubules (e.g. single arrowhead) and an early phragmoplast (double arrowhead) can be seen. Scale: **A**: X380; **B**: X260; **C**: X330; **D**: X670.

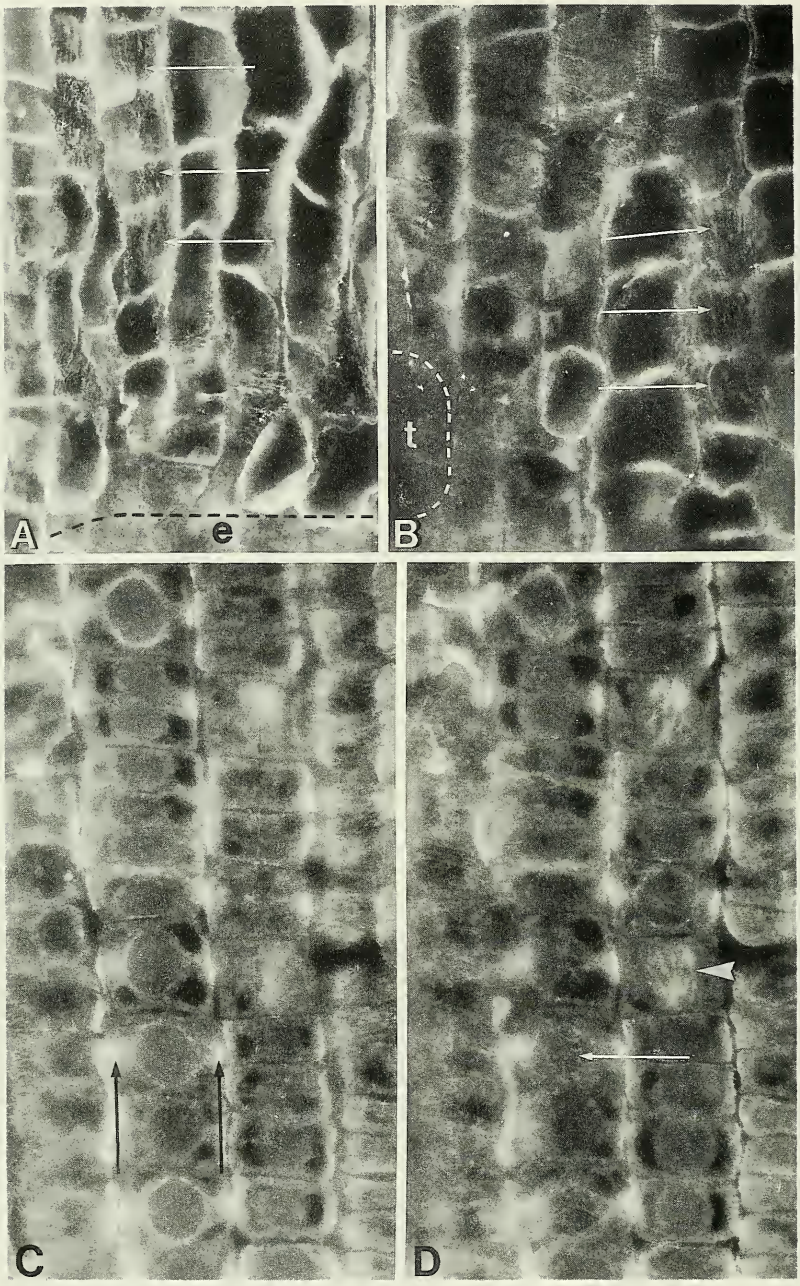

Fig. 2. Immunofluorescence micrographs of immunolabelled microtubules in *P. sativum* root cells. Continuous alignment of microtubules (e.g. arrows) is visible across files of cells in **A** and **B**, which are close to the edge (e, **A**) or tip (t, **B**) of a wedge-shaped wound. **C** and **D** show files of cells in the meristematic zone of an intact root tip, but in different focal planes: in one focal plane (**C**), some cells clearly exhibit pre-prophase bands of microtubules (e.g. arrow) and in another focal plane (**D**), a further array of microtubules arranged in a network across the nucleus (arrow), is revealed in the same cell. Also in the focal plane illustrated in **D**, a different cell displays a microtubule spindle (arrowhead) which was not seen in the previous plane of focus. Scale: **A**: X280; **B**: X300; **C, D**: X630.

and its orientation and position predicts the exact position at which the developing cell plate will attach during late telophase (for review, see Gunning, 1982).

Since this method minimizes disruption of files of cells and tissue fragmentation, it allows visualization of the continuous alignment of microtubules across neighbouring cells, where this occurs in tissue. One such instance in which this does occur in plant tissues is in cells near a wound, where changes in the orientation of cortical microtubules occur shortly after wounding. Figures 2A and B show two examples of wounded pea root tissue where the orientation of microtubules can be seen to extend across files of adjacent cells close to the edge (Fig. 2A) and tip (Fig. 2B) of a wedge-shaped wound, made by cutting and removing a piece of tissue from the root with a microscalpel. (A detailed description of wound-induced changes in microtubule orientation and its role in organized wound repair is described in Hush et al., 1990).

Further, this technique allows three-dimensional information about microtubule arrays to be elucidated. For instance, in the same cells illustrated in Figures 2C and D, different microtubule arrays can be detected in the two different focal planes. In one cell (C, arrows), a pre-prophase band is seen in section, but in another focal plane, a different network of microtubules can be distinguished around the nucleus (D, arrow). In larger, non-meristematic cells, this sort of three-dimensional information can also be gained from sections which are approximately the same width as the cells being examined, by recording photographically the microtubule orientations in different focal planes.

Discussion

This new technique for the visualization of microtubules of cells *in situ* by improved methods of tissue embedding and infiltration, as well as slide preparation, provides a useful experimental protocol where existing methods for thick sectioning of large areas of tissues were not adequate.

A variety of microtubule arrays in meristematic, elongating and differentiating cells were readily visualized using this technique. In elongating, interphase cells up to 3.5 cm from the root tip, transverse microtubule arrays were found to predominate. Hogetsu and Oshima (1986) have also observed transverse cortical microtubules 0-4 mm from the tip in pea roots, and they showed that about 5 mm from the tip, at which point cell elongation had ceased, microtubules in cortical cells were oriented obliquely. These observations are consistent with our findings of oblique cortical microtubules in differentiating and fully mature cells.

It is particularly useful to be able to build a three-dimensional picture of microtubule arrays in plant cells, since it is the precise spatial organization of microtubules throughout plant cells which is responsible for the directional control that microtubules can exert on plant cells during growth and development. Such three-dimensional information about microtubule arrays can clearly be attained by recording microtubule alignments in different focal planes, as has been described here. For very large cells such as those beyond the zones of division and elongation, the same approach could be applied to serial sections. With reference to this, a future application of this technique could be to examine specimens prepared by this method using confocal scanning optical microscopy rather than convential light microscopy. The advantage of confocal microscopy is that non-invasive serial optical sections can be made through very precise focal planes of cells, and the out-of-focus blur which usually accompanies a conventional microscopic image is greatly reduced, producing very high-resolution images (for review of the application of confocal microscopy to biological specimens, see Shotton, 1989). Furthermore, confocal microscopy could yield a very precise picture of the three-

dimensional distribution and arrangement of microtubules in cells by subsequent image processing techniques such as three-dimensional reconstruction of optical sections through a single cell or tissue.

One aspect in which the technique described in this paper is especially successful, is that it enables the alignment of microtubules along files of neighbouring cells to be distinguished. This is of particular importance since current research to elucidate the mechanism by which microtubules are oriented in plant cells, presently focusses on the possibility that microtubules align with respect to endogenous physical fields within plant cells and whole tissues (Hush et al., 1990; Hush and Overall, 1991; Williamson, 1990, 1991). In summary, this technique readily allows the immunolocalization of microtubules across neighbouring cells *in situ*. This may lead to a more comprehensive understanding of the way in which microtubules can exert precise control on the spatial development of plant cells and the morphogenesis of plant tissues and organs.

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